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Adenomatous polyposis coli (APC)-induced apoptosis of HT29 colorectal cancer cells depends on mitochondrial oxidative metabolism☆,☆☆

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ABSTRACT

Adenomatous polyposis coli (APC) is a tumor suppressor involved in the Wnt signaling, the primary driving force of the intestinal epithelium homeostasis. Alterations of components of the Wnt pathway, and in most cases mutations of APC, have been reported to promote colorectal cancer (CRC). During differentiation the enterocytes migrate from the crypt to the tip of the villus where they undergo apoptosis thus ensuring the continual renewal of the intestinal mucosa. The differentiation process is characterized by an activation gradient of the Wnt signaling pathway accompanied by a metabolic switch from glycolysis to mitochondrial oxidative phosphorylation along the crypt–villus axis. In the present work, we study the relationship between the expression of wild type APC protein and mitochondrial oxidative metabolism in HT29 colorectal cancer cells, originally carrying endogenous inactive *APC* alleles. By generating mtDNA-depleted (rho0) APC-inducible HT29 cells, we demonstrate for the first time that the APC-dependent apoptosis requires the production of reactive oxygen species (ROS) by the mitochondrial respiratory chain. The possible role of mitochondria as putative target in the prevention and/or therapy of colorectal cancer is herein discussed.

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1. Introduction

Wnt signaling is the main pathway involved in the regulation of the intestinal epithelium homeostasis and differentiation along the crypt-villus axis [1]. This pathway includes the *Adenomatous polyposis coli* (APC) tumor suppressor gene product involved in the scaffolding of a so-called "destruction complex", that destabilizes and controls the turnover of β-catenin [2].

Under physiological conditions, intestinal epithelial cells migrate from the bottom of the crypt to the top of the villus where they differentiate from progenitor to mature enterocytes and lose their proliferative activity [3]. In this context, if the pathway is active, β-catenin translocates into the nucleus where it binds the LEF/TCF transcription factors and induces the expression of genes involved in the cellular proliferation, such as *c-Myc* [4] and *cyclin D1* [5]. In the absence of Wnt ligands, β-catenin is phosphorylated by the destruction complex and targeted for ubiquitine-dependent proteasomal degradation [6]. Indeed, a gradient of activation of the Wnt pathway occurs along the crypts with the progenitor cells located at the bottom showing the highest proliferative activity in response to the accumulation of nuclear β-catenin [1].

Aberrant Wnt pathway activation is considered the first event in intestinal tumorigenesis, with other mutations being required to facilitate

Abbreviations: APC, adenomatous polyposis coli; CDX2, caudal-related homeobox 2; COX, cytochrome *c* oxidase; CRC, colorectal cancer; DNP, 2,4-dinitrophenol; FAP, familial adenomatous polyposis; HT29 APCi, HT29 APC inducible; HT29 βGal, HT29 β galactosidase; IRS1, insulin receptor substrate 1; KLF4, Kruppel-like factor 4; mt-COII, mitochondrial cytochrome *c* oxidase II; NAC, N-acetyl-L-cysteine; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TMPD, *N,N,N′,N′*-tetramethyl-p-phenylenediamine dihydrochloride; Tfam, mitochondrial transcription factor A; $ZnCl_2$, zinc chloride

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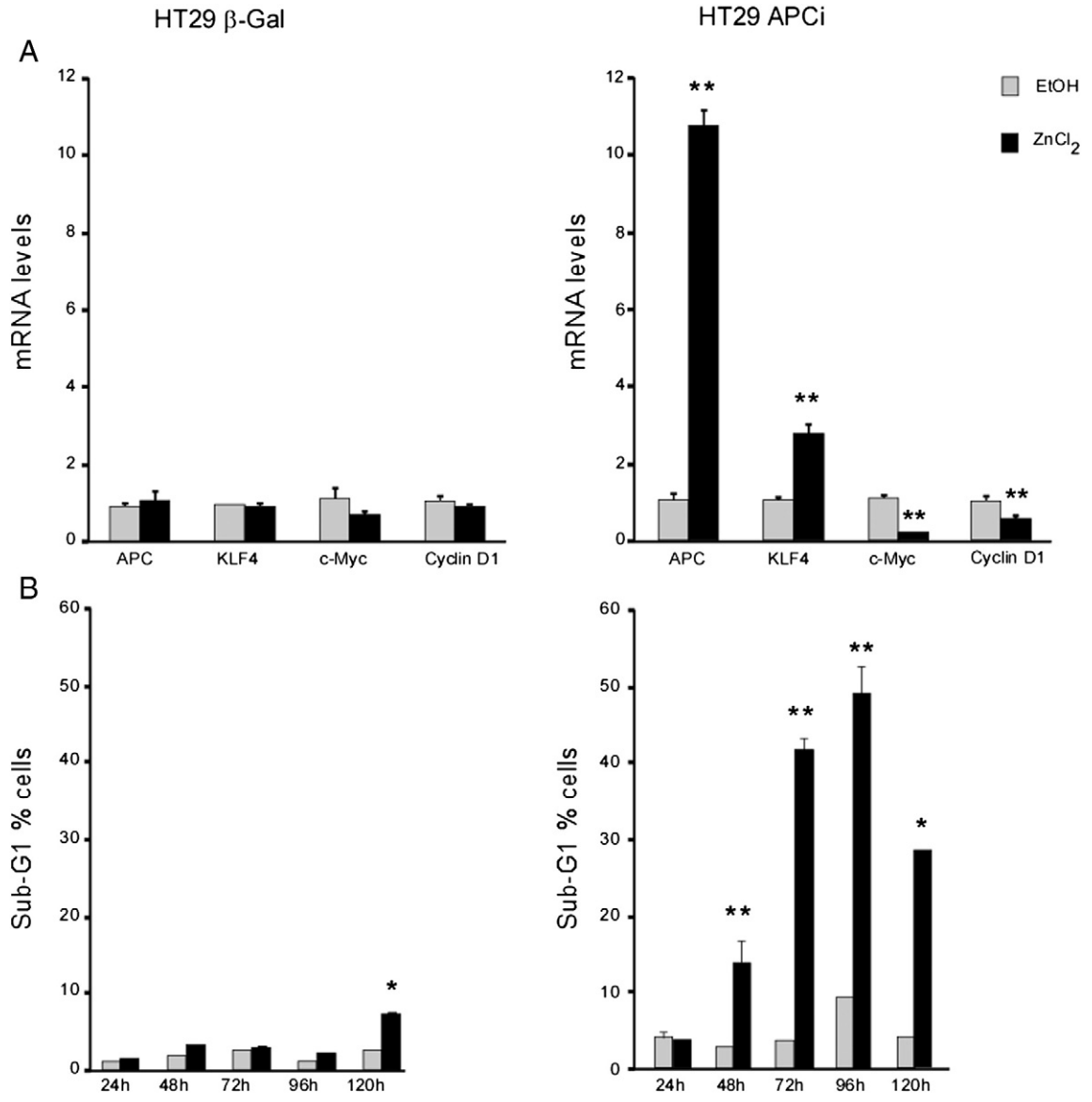

Fig. 1. Expression of the wild type APC restores the Wnt signaling and promotes apoptosis in HT29 cells. Expression of wild type APC or β-Gal gene was induced by $ZnCl_2$ (120 μM) and cells were harvested and analyzed at the indicated time points. *$p < 0.05$; **$p < 0.01$ for $ZnCl_2$-treated cells vs. vehicle (EtOH)-treated controls. (A) Expression levels of *APC*, *KLF4*, *c-Myc* and *Cyclin D1* genes were analyzed after 24 h by RTqPCR. Values represent mean ± SEM for two independent experiments performed in triplicate. *GAPDH* was used as reference gene. (B) The percentage of the sub-G1 phase cells was measured by flow cytometry. Values shown represent mean ± SEM for two independent experiments performed in duplicate.

the progression from adenomas to malignant, invasive and metastatic cancers [7]. Usually, germinal mutations in the *APC* gene underlie the development of large numbers of colon polyps, or adenomas, in Familial Adenomatous Polyposis (FAP) patients. Approximately 85% of all sporadic and hereditary colorectal tumors is characterized by the loss of APC. *APC* is considered a classic tumor suppressor gene as both alleles must be inactivated for loss of tumor suppressing activity [7]. For CRC and FAP patients, the molecular mechanisms underlying the lack of APC protein can be a second truncating mutation or an allelic loss of the second allele, representing the limiting step for tumor initiation [8]. Moreover, expression of the full length *APC* gene, in HT29 human colorectal cancer cells containing endogenous inactive APC alleles, promotes apoptosis [9].

The loss of proliferative activity and the differentiation process of the intestinal epithelium along the crypt-villus axis is also associated with a metabolic gradient, with crypt producing ATP primarily through glycolysis and differentiated cells depending on mitochondrial oxidative phosphorylation (OXPHOS). As a result of the increased demand in mitochondrial activity [10], the differentiated epithelial cells at the apex of the villus undergo apoptosis [11], mostly via mitochondrial ROS production [12,13], thus guaranteeing the continual renewal of the intestinal mucosa.

Mitochondria are the powerhouses of aerobic eukaryotic cells. They contain their own genome [14] (~16.6 kb) encoding 22 tRNAs, 2 rRNAs and 13 protein subunits [15] belonging to the OXPHOS machinery. As a by-product of the electron flux through the respiratory chain, mitochondria produce the majority of cellular ROS [16]. Besides their central role in cellular bioenergetics, mitochondria are also involved in the complex scenario of programmed cell death [17,18]. In fact, the release of the electron carrier protein cytochrome c from the intermembrane space to the cytoplasm is one of the primary activating steps of the apoptotic caspase cascade [19,20].

The possible interaction between APC and mitochondria in relation to the apoptotic process has been previously investigated [9,21,22], yet nothing is known of their mechanistic relationship. In order to understand the functional link between mitochondrial oxidative metabolism and Wnt signaling in intestine cell fate, we have generated HT29 APC inducible cells depleted of their mitochondrial DNA (rho0 cells), and thus lacking a functional respiratory chain and OXPHOS

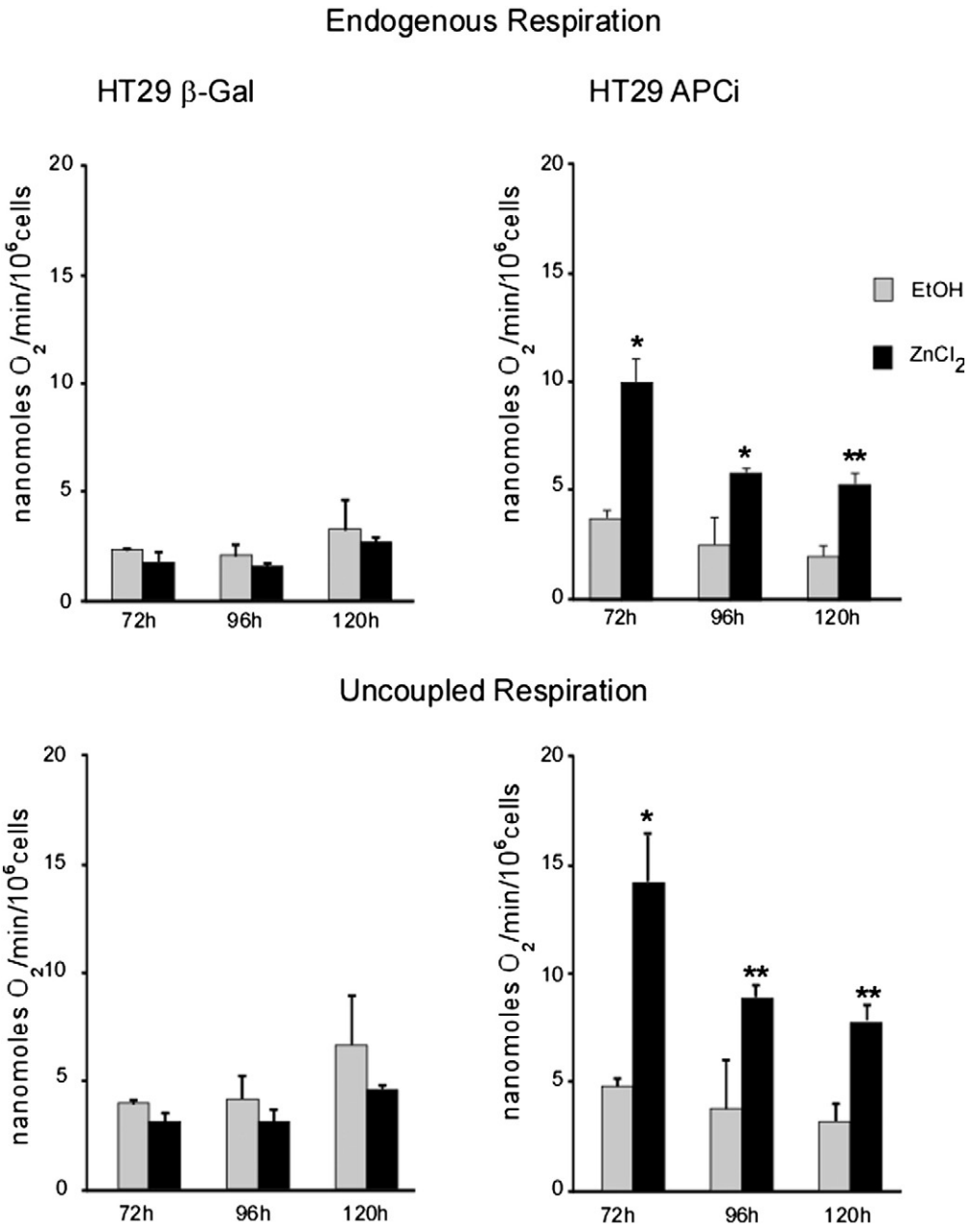

Fig. 2. APC expression increases mitochondrial respiratory fluxes in HT29 cells. Expression of wild type APC or β-Gal gene was induced by $ZnCl_2$ (120 μM) and cells were harvested and analyzed at the indicated time points. *$p < 0.05$; **$p < 0.01$ for $ZnCl_2$-treated cells vs. vehicle (EtOH)-treated controls. Basal and DNP-uncoupled endogenous respiration rates are expressed as nanomoles O_2/min/10^6 cells. Values shown represent mean ± SEM for two independent experiments performed in triplicate.

activity [23]. In this cell model, we show that the induced expression of the full length APC protein fails to stimulate apoptosis uncovering, for the first time, that this effect requires mitochondrial respiratory ROS production. Our results provide further insight on the pivotal role of mitochondria in intestinal cell differentiation and suggest the possibility to consider mitochondria as a possible target for the prevention and/or the treatment of colorectal cancer.

2. Methods

2.1. Cell line and culture conditions

APC-inducible (HT29 APCi) and β galactosidase-inducible (HT29 βGal) HT29 cell lines were kindly provided by Dr Bert Vogelstein [9]. The cells were cultivated in McCoy's 5A media (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 0.6 mg/ml hygromicin B (Life Technologies) at 37 °C in a humidified atmosphere of 5% CO_2. The expression of the inducible genes was initiated with 120 μM zinc chloride ($ZnCl_2$; Sigma-Aldrich).

The mtDNA-depleted ρ0 cell lines were generated by exposure to ethidium bromide (EtBr; Promega) [23]. Briefly, HT29APCi and HT29 βGal cells were cultured for 40 days in McCoy's 5A media supplemented with 10% FBS, 0.2 μg/ml of EtBr, 100 μg/ml pyruvate and 50 μg/ml uridine, without antibiotics. After assessment of the absence of mtDNA, HT29 ρ0, HT29 APCi ρ0 and HT29 βGal ρ0 cells were cultured in McCoy's 5A media supplemented with 10% FBS, 1% penicillin/streptomycin, 0.6 mg/ml hygromicin, 100 μg/ml pyruvate, and 50 μg/ml uridine.

For treatment with the antioxidant N-Acetyl-L-Cysteine (NAC; Sigma-Aldrich), NAC was dissolved in serum-free culture medium and added to a final concentration of 20 mM. HT29 APCi, HT29 βGal, HT29 APCi ρ^0 and HT29 βGal ρ^0 cells were seeded in 6-well plates (Falcon) at a density of

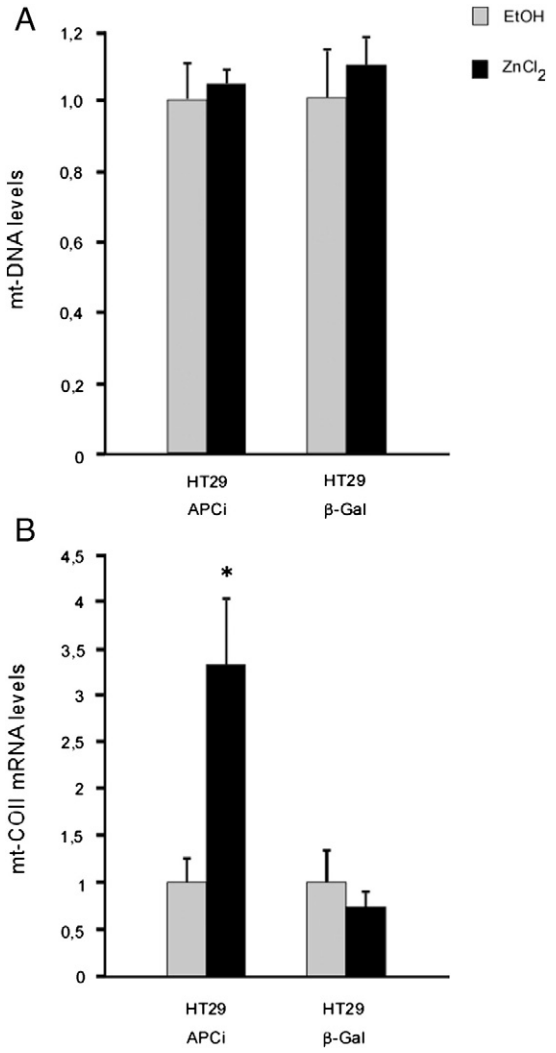

Fig. 3. APC induction increases expression of cyt c oxidase subunit II gene (mt-COII) in HT29 cells. (A) Determination of the relative mtDNA level in HT29 APCi and in HT29 β-Gal cells upon $ZnCl_2$-induced APC expression. Cells were analyzed for relative mt-COII levels by real-time qPCR, and actin was used as a nuclear reference gene. (B) mt-COII mRNA levels were measured in HT29 APCi and in HT29 β-Gal cells by real-time qPCR. GAPDH was used as housekeeping gene. Values shown represent mean ± SEM for two independent experiments performed in triplicate. *$p < 0.05$; **$p < 0.01$ for $ZnCl_2$-treated cells vs. vehicle (EtOH)-treated controls.

0.5×10^6 cells per well, pretreated with NAC for 2 h in serum-free medium, and then induced with 120 μM $ZnCl_2$ in normal growth medium.

2.2. Measurement of mtDNA by quantitative Real-Time PCR (RTqPCR) analysis

After APC induction, mtDNA was quantified by RTqPCR. RTqPCR primers were designed using Primer Express software. Briefly, cells were harvested and total DNA was extracted using the *DNeasy Tissue Kit* (Qiagen) according to the manufacturer's instruction. 60 ng of total DNA was used in realtime PCR analysis performed in 96 well optical reaction plates using the ABI 7500HT machine (Applied Biosystem). The PCR was performed with primers for the mitochondrial encoded cytochrome *c* oxidase II (mt-COII) gene. PCR assays were conducted in triplicate wells for each sample. Baseline values of amplification plots were set automatically and threshold values were kept constant to obtain normalized cycle times and linear regression data. The following reaction mixture per well was used: 10 μl Power Syber Green (Applied Biosystem), 2.4 μl of primers at the final concentration of 150 nM, 4.6 μl sterile water, and 3 μl total DNA (60 ng). For all experiments the following PCR conditions were used: denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec then at 60 °C for 60 s. Quantitative normalization of mtDNA in each sample was performed using actin as internal control.

2.3. Detection of mtDNA depletion by multiplex PCR analysis

Total DNA was isolated from the indicated cell lines using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instruction, and used as a template in a multiplex PCR test. The genomic DNAs isolated from HT29APCi, HT29 βGal and HT29ρ0 cell lines were used as positive and negative controls, respectively. The PCR was carried out with primers for the mitochondrial cytochrome b gene (forward-5′ tacaaccacgaccaatgatatgaaa 3′ and reverse-5′ aggttttcatcatctccggtttacaag 3′) and the nuclear actin gene (forward-5′ tgactgactcctcatgaagatc 3′ and reverse-5′ ccgtcaggcagctcgtagctct 3′) using the ABI 7500HT machine (Applied Biosystem, Life Technologies). The annealing temperature was 55 °C for 30 cycles with an elongation time of 45 s.

2.4. RNA extraction

Total RNA was isolated by QIAzol reagent (Qiagen) following the manufacture's instruction. To avoid possible DNA contaminations, RNA was treated with DNAase-1 (Ambion, Life Technologies). cDNA was synthesized reverse-transcribing 4 μg of total RNA in a total volume of 100 μl using the High Capacity DNA Archive Kit (Applied Biosystem, Life Technologies) following the manufacturer's instruction.

2.5. Quantitative Real-Time PCR (RTqPCR)

RTqPCR primers were designed using the Primer Express software. PCR assays were performed in 96 well optical reaction plates using the ABI 7500HT machine (Applied Biosystem, Life Technologies). PCR assays were conducted in triplicate wells for each sample. Baseline values of amplification plots were set automatically and threshold values were kept constant to obtain normalized cycle times and linear regression data. The following reaction mixture per well was used: 10 μl Power Sybr Green Kit (Applied Biosystem, Life Technologies), 2.4 μl of primers at the concentration of 150 nM, 4.6 μl RNAse free water, and 3 μl cDNA (60 ng). For all experiments the following PCR conditions were used: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec then at 60 °C for 60 sec. Quantitative normalization of cDNA in each sample was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Relative quantification was performed using the $\Delta\Delta C_T$ method. Validated primers for RTqPCR are available upon request.

2.6. Flow cytometry for analysis of cell viability

Attached and floating cells were harvested and suspended in phosphate buffered saline (PBS; Gibco, Life Technologies). Therefore, cells were stained with 50 μg/ml propidium iodide (PI; Sigma-Aldrich), 0.5 μg/ml RNase (Sigma-Aldrich) and 1% Nonidet P-40 (Sigma-Aldrich) and incubated in the dark at room temperature for 1 h. Sub-G1 phase cells were detected using the FACSVantage flow cytometer (BD Bioscience) and analyzed by the Cell Quest-PRO software (BD Bioscience). Human lymphocytes were used as a control.

2.7. Measurements of ROS in HT29APCi and HT29 APCi ρ^0 cells

The intracellular ROS levels were determined using the cell-permeant CM-H_2DCFDA [5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate] fluorescent probe (Molecular Probes, Life Technologies). Cells were dissociated with trypsin (Gibco, Life Technologies), washed, and then incubated in PBS and 4 μM CM-H_2DCFDA. As negative control, cells were incubated in PBS and vehicle (DMSO). As positive control, cells were incubated with PBS, 4 μM CM-H_2DCFDA and 8 mM H_2O_2. After incubation, in the dark, for 20 min at 37 °C, cells were washed with PBS, and

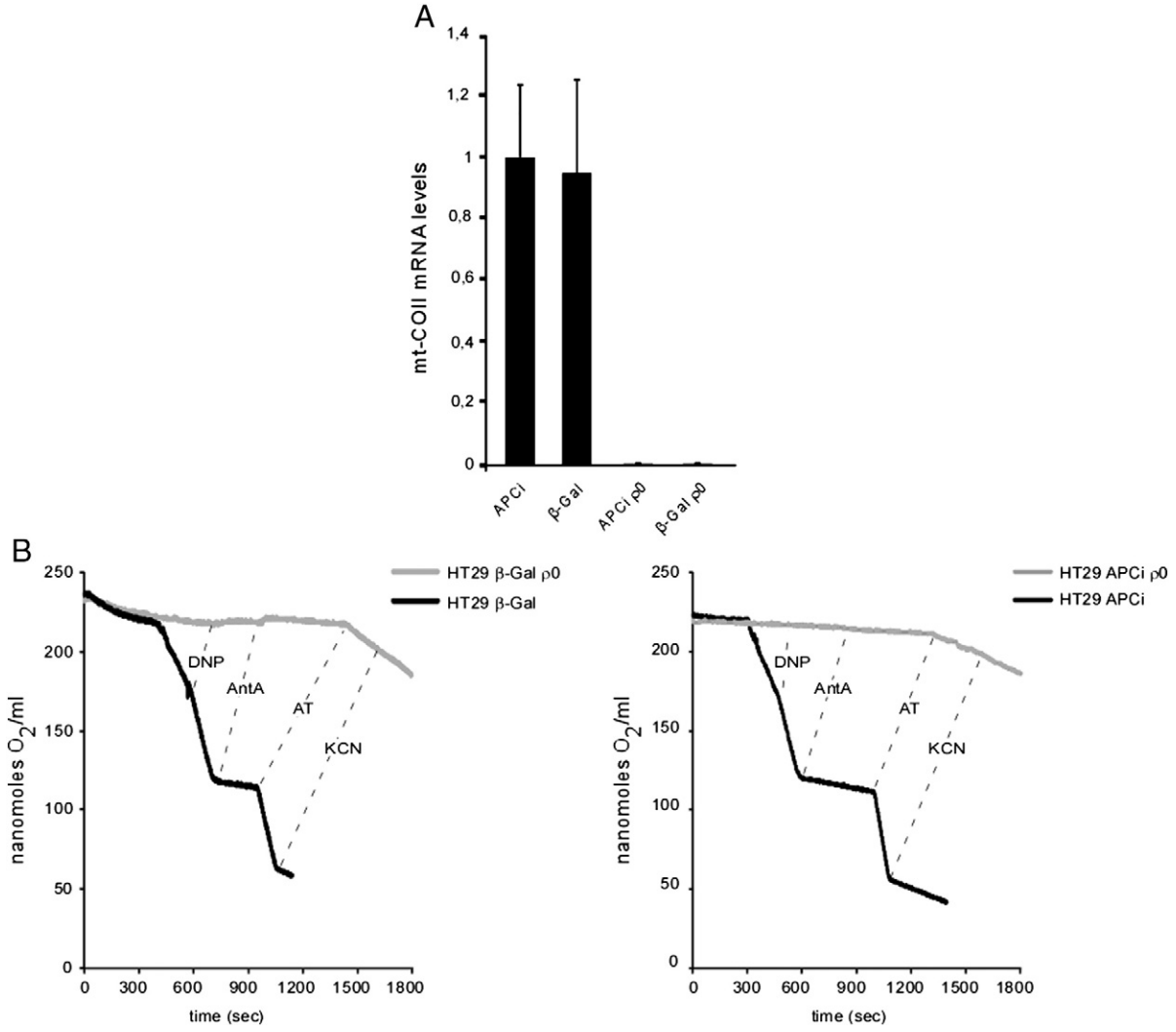

Fig. 4. Characterization of mtDNA-depleted (ρ0) HT29 APCi and HT29 β-GAL cells. (A) Expression of mitochondrial cytochrome c oxidase II gene before and after mt-DNA depletion. (B) Representative oxygraphic tracings of respiratory fluxes in intact cells. DNP, 2,4-dinitrophenol (60 μM); Ant A, antimycin A (40 nM); AT, ascorbate (10 mM) plus *N,N,N′,N′*-tetramethyl-p-phenylenediamine (0.4 mM); KCN, potassium cyanide (2 mM).

then the fluorescence emission of the cell suspensions in PBS was assessed by flow cytometry.

2.8. Assessment of mitochondrial respiratory rates in intact cells

The respiratory activity of intact cells was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber (Hansatech Instruments), magnetically stirred at 37 °C, essentially as previously described [24]. Briefly, exponentially growing cells were collected by trypsinization and centrifugation, and then transferred into the polarographic chamber at 4–6 × 10^6 cells/ml in TD buffer [0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris–HCl (pH 7.4)], previously air-equilibrated at 37 °C. The respiration rates by endogenous substrates were evaluated directly (basal endogenous respiration), after addition of 2,4-dinitrophenol (DNP) to a final concentration of 60 μM (uncoupled maximal endogenous respiration) and after addition of sodium ascorbate (10 mM) + *N,N,N′,N′*-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.4 mM) as artificial membrane-permeant electron donors to cytochrome *c* oxidase (COX), in the presence of the upstream respiratory chain inhibitor antimycin A (40 nM). Due to the biphasic response of the cellular respiration to DNP, a DNP titration was carried out in HT29 APCi cells to choose the optimal DNP concentration as the minimal one resulting in the maximal stimulation of the respiration rate.

2.9. Statistical analysis

All results are expressed as mean ± SEM. Data distribution and gene expression statistical analysis were performed using NCSS statistical and power analysis software 2007 (Kaysville, Utah, USA). Comparisons of two groups were performed using a Student's t test followed by Mann–Whitney U test where appropriate. A $p < 0.05$, $p < 0.01$ were considered significant.

3. Results

3.1. APC expression restores Wnt signaling and promotes apoptosis in HT29 APCi cells.

HT29 human colorectal cancer cells produce two carboxyl-terminal truncated APC proteins of approximately 100 kDa and 200 kDa, respectively. This cell line was stable transfected with vectors for $ZnCl_2$-inducible [25,26] expression of wild type APC (HT29 APCi) and β-Gal (HT29 β-Gal) genes, respectively [9,12]. As previously reported [9], the expression of the full length APC restored the physiological regulation of the Wnt pathway in HT29 APCi cells as revealed by RTqPCR analysis of the *KLF4* and *c-Myc* marker gene expression (Fig. 1A). In fact, the induction of APC by $ZnCl_2$ caused an increase in the mRNA level of *KLF4*,

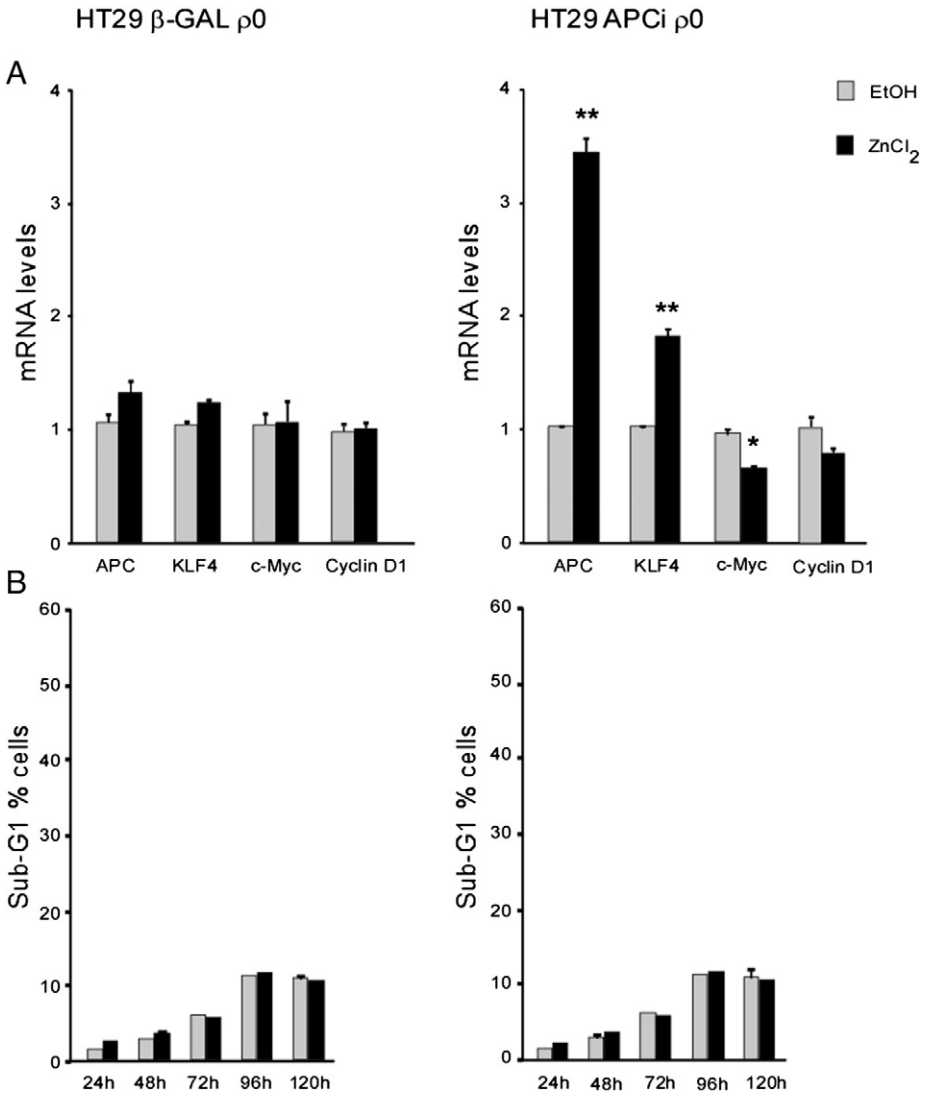

Fig. 5. Effects of the induction of wild type APC expression in HT29 APCi ρ0 cells. (A) Expression levels of *APC*, *KLF4*, *c-Myc* and *Cyclin D1* genes. Cells were harvested 24 h after $ZnCl_2$ exposure and relative mRNA levels were quantified by RTqPCR on RNA samples using GAPDH as reference gene. Values shown represent mean ± SEM for two independent determinations performed in triplicate (*$p < 0.05$; **$p < 0.01$). (B) Time course analysis of apoptotic cell population after induction of wild type APC expression. Expression of wild type APC or β-Gal gene was induced by $ZnCl_2$ (120 μM) and cells were harvested and analyzed by flow cytometry at the indicated time points. Values shown represent mean ± SEM for two independent determinations performed in duplicate. (*$p < 0.05$; **$p < 0.01$).

an indirect target of APC activated through Caudal Type Homeobox transcription factor 2 (*CDX2*) [27], a significant decrease in the expression of *c-Myc*, a β-catenin target gene [4] and *cyclin D1*, a target for the β-catenin/LEF-1 complex [28] as compared to vehicle (EtOH)-treated cells. As expected, the expression levels of the same genes were unchanged in HT29 β-Gal control cells.

To confirm the ability of the cells to undergo apoptosis upon APC induction, we measured the sub-G1 phase cell population by flow cytometric analysis. As shown in Fig. 1B, after 48 h of APC induction by $ZnCl_2$ a significant increase in the sub-G1 cell content could be observed in APCi, but not in β-Gal cells when compared to mock-treated controls. The relative increase in sub-G1 cells reached its maximum (about 50%) after 96 h, but was still significantly present at 120 h from $ZnCl_2$ exposure.

3.2. Induction of APC increases mitochondrial respiratory fluxes in HT29 cells

Along the crypt–villus axis, intestinal cells show increasing levels of the APC protein, as well as a higher respiratory capacity measured via COX activity [12]. To identify a direct link between APC induction and mitochondrial functions, we measured the respiratory capacities by endogenous substrates in HT29 APCi and HT29 β-Gal intact cells (Fig. 2). A significant enhancement of both the basal and the maximal uncoupled endogenous respiratory fluxes was detected upon $ZnCl_2$-induced APC expression in APCi cells, but not in β-Gal cells, as compared with their control cells, respectively. These data, while confirming that the expression of the full length APC protein restores a normal Wnt signaling pathway and promotes apoptosis, also suggest that is associated with an increase in mitochondrial respiration.

3.3. APC expression induces mitochondrial transcription

To investigate whether the induction of APC could result in an increase in mtDNA content, the total genomic DNAs of the HT29 APCi and HT29 β-Gal cells, were used as templates in a RTqPCR to amplify the mitochondrial cytochrome c oxidase II gene (mt-COII). As shown in Fig. 3A, levels of mtDNA remain unchanged upon $ZnCl_2$-induced APC expression in APCi cells and in β-Gal cells. On the other hand, in order to check whether APC induction could influence mtDNA gene expression, the same RTqPCR analysis of mt-COII gene, was carried out on total RNA from the same cells. We found that the expression of mt-COII

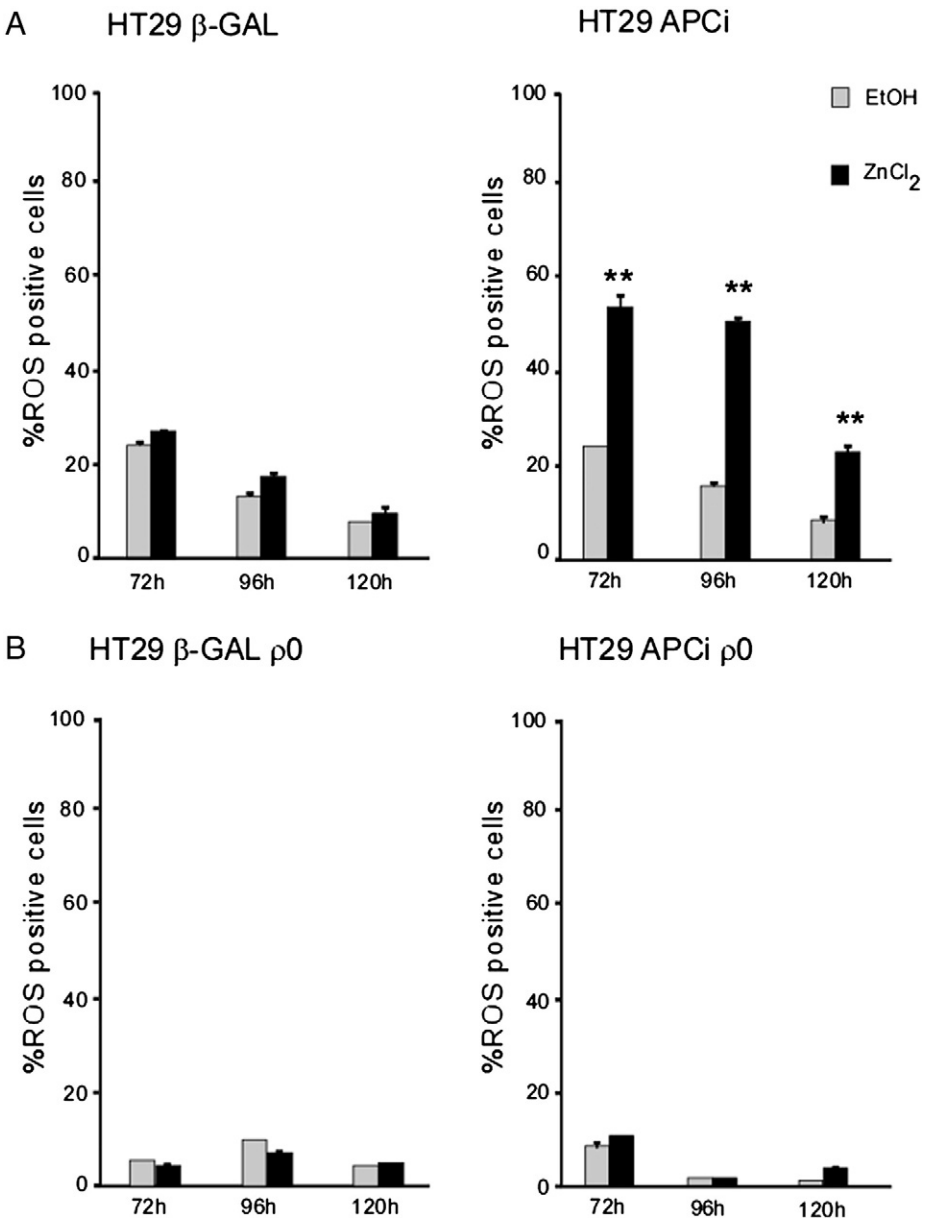

Fig. 6. Mitochondrial respiratory chain is needed for the APC-induced increase in ROS production. Expression of wild type APC or β-Gal gene was induced by $ZnCl_2$ (120 μM) and cells were harvested and analyzed at the indicated time points. *$p < 0.05$; **$p < 0.01$ for $ZnCl_2$-treated cells vs. vehicle (EtOH)-treated controls. The intracellular ROS levels were determined using the cell-permeant fluorescent probe CM-H_2DCFDA, and the percentage of ROS positive cells was quantified by flow cytometry. Values shown represent mean ± SEM for two independent determinations performed in duplicate. (*$p < 0.05$; **$p < 0.01$).

gene results increased upon $ZnCl_2$-induced APC expression in APCi cells, but not in β-Gal cells. These data point to an activated mitochondrial transcription due to inducible expression of APC (Fig. 3B).

3.4. Mitochondrial DNA-depletion (Rho0) in HT29 APCi and HT29 β-Gal cells

To confirm that the increase in mitochondrial respiratory activity could represent the functional link between the restored APC function and the subsequent stimulation of the apoptotic process, we generated mtDNA-depleted HT29 APCi ρ^0 and HT29 β-Gal ρ^0 cell lines by ethidium bromide treatment [23]. The complete removal of mtDNA was verified by the absence of any amplification product of the mtDNA cytochrome b gene (*cyt b*) (Fig. S1) in both HT29 APCi ρ^0 and HT29 β-Gal ρ^0 cells. In addition, in the same cells, we verified the lack of mt-COII mRNA (Fig. 4A), further confirming the mtDNA depletion. To characterize the ρ^0 state at a functional level, we measured mitochondrial respiratory fluxes. As expected, contrary to the parental counterparts, no antimycin-sensitive respiration was detected in the intact ρ^0 cells. Similarly, no COX-specific activity was present in both ρ^0 cell lines as revealed by the absence of KCN-sensitive oxygen consumption elicited by the artificial membrane-permeant cytochrome c reductant ascorbate + TMPD (Fig. 4B).

We then checked if APC induction by $ZnCl_2$ would restore a normal Wnt pathway in HT29 APCi ρ^0 cells. As shown in Fig. 5A, the $ZnCl_2$-induced changes of the expression levels of *APC*, *KLF4*, *c-Myc* and *cyclin D1* marker genes were maintained (see also Fig. 1B) in HT29 APCi ρ^0 cells, thus demonstrating that the absence of mtDNA does not interfere with the normal regulation of the transcriptional program dependent on the APC/β-catenin pathway.

Finally, we determined whether the proapoptotic effect of the APC expression was also present in HT29 APCi ρ^0 cell line (Fig. 5B). Indeed, it has been previously demonstrated that ρ^0 cells can undergo apoptosis [29] and that mtDNA depleted mitochondria can mediate the cell death

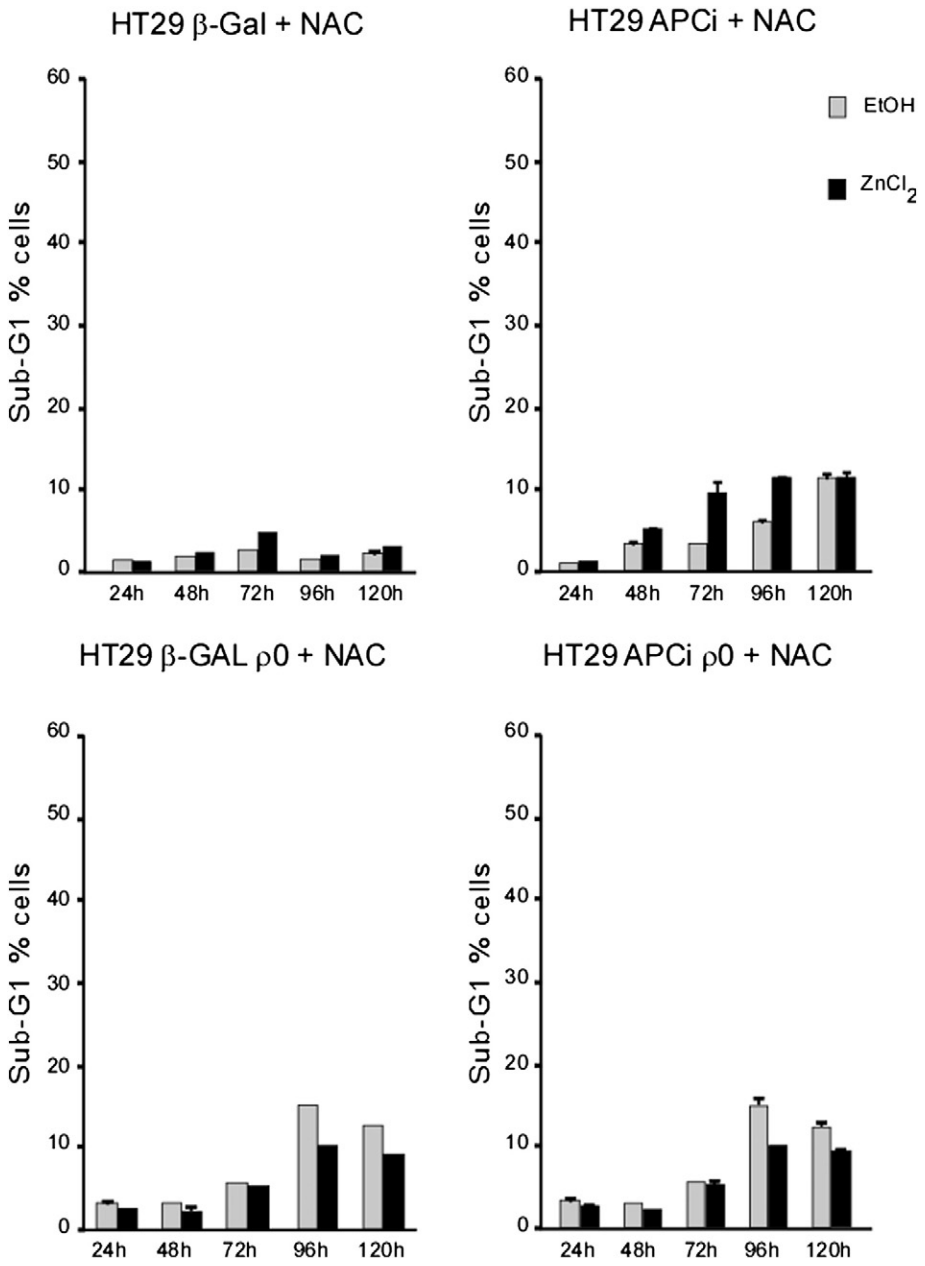

Fig. 7. Loss of APC-dependent apoptosis in NAC-treated HT29 cells. Expression of wild type APC or β-Gal gene was induced by $ZnCl_2$ (120 μM) and cells were harvested and analyzed at the indicated time points. *$p < 0.05$; **$p < 0.01$ for $ZnCl_2$-treated cells vs. vehicle (EtOH)-treated controls. Apoptotic cells were quantified as percentage of sub-G1 phase cells by flow cytometry as previously described. $ZnCl_2$-induction of APC expression was initiated after a 2 h pre-incubation with the antioxidant NAC (20 mM). Values represent mean ± SEM for two independent determinations performed in duplicate. (*$p < 0.05$; **$p < 0.01$).

process by releasing cytochrome c [30]. In particular, the occurrence of the cell death process in response to different apoptotic stimuli has been studied in a variety of cancer cell lines [31,32]. Interestingly, no difference was found in the time course analysis of the subG1 phase cell content when comparing $ZnCl_2$-treated APCi ρ^0 cells to their mock-treated controls or to β-Gal ρ^0 cells, respectively. These results suggest a possible link between the APC-dependent apoptosis, and the presence of a functional mitochondrial respiratory chain.

3.5. APC-induced apoptosis requires mitochondrial respiratory ROS.

We have previously demonstrated that one of the main apoptosis-driving factors in colorectal cancer cells is represented by the mitochondrial respiratory chain-dependent accumulation of ROS [12]. To test the hypothesis that the mitochondrial oxidative burst could have been the missing link between the restored Wnt pathway and apoptosis in APCi ρ^0 cells, we measured ROS accumulation upon APC induction in the ρ^0 cell lines and their parental counterparts. As shown in Fig. 6, a significant increase in the percentage of ROS-positive cells could be observed only in HT29 APCi cells, when comparing $ZnCl_2$-treated cells to the mock-treated controls or their corresponding ρ^0 lines, respectively. It is worth noting that the basal content of ROS positive cells was always lower in ρ^0 cells as compared to their parental cell lines, thus pointing to the mitochondrial respiratory chain as the main producer of ROS under our experimental conditions.

Finally, pre-treatment of the cells with the anti-oxidant N-acetyl-L-cysteine (NAC) almost completely abolished the increase in apoptotic cells in APCi cells upon $ZnCl_2$-induced APC expression (Fig. 7

cfr. Fig. 1B), thus confirming the direct functional relationship between the mitochondrial oxidative burst and the proapoptotic action of APC.

4. Discussion

Mitochondria, in addition to their main task of producing aerobic energy through the OXPHOS apparatus, are increasingly shown to be involved in a variety of metabolic and signaling pathways of eukaryotic cells. We have recently demonstrated that mitochondria play a pivotal role in intestinal cell fate, not only by regulating the metabolic switch during cellular differentiation, but also by modulating the abundance of ROS production as the main driving signal for the apoptotic renewal of intestinal epithelial cells [12,33].

In this paper, we identify a functional link between mitochondria and Wnt signaling, i.e., the major signal transduction pathway involved in intestinal cells homeostasis and development along the crypt–villus axis. By generating a mtDNA-depleted APCi HT29 cell line, we clearly demonstrate that a functional respiratory chain is required for the proapoptotic effect associated with the expression of the full length APC protein, hence, with the re-establishment of a normal Wnt pathway, in colorectal cancer HT29 cells. Indeed, a direct interaction between the caspase cleaved amino-terminus of APC and the mitochondrial protein hTID-1 has been previously proposed to enhance sensitivity to apoptosis in colorectal cancer cells [22].

On the other hand, our results are apparently in contrast with previous reports of an increased Wnt signaling as a potent activator of mitochondrial biogenesis and respiratory capacity, as well as, of ROS production, via induction of the insulin receptor substrate-1 (*IRS1*) and *c-Myc* gene expression in C2C12 muscle cells and primary mouse embryonic fibroblasts [34]. In fact, the transcription factor c-Myc, known to be an important activator of mitochondrial biogenesis and function [35,36], is negatively regulated upon APC induction in HT29 colorectal cancer cells (Fig. 1B), thus pointing to a striking tissue-specificity regulation of the Wnt/IRS1/c-Myc pathway, in relation to mitochondrial activity. Interestingly, in line with our findings, IRS1 and c-Myc have been shown to be overexpressed in colorectal cancer as compared with paired normal mucosa samples [37]. Therefore, mitochondria seem to be differently regulated in the intestine, where OXPHOS and respiratory ROS-dependent apoptosis are required for epithelial cell differentiation and self-renewal, respectively, along the crypt to villus axis [12].

Nevertheless, the role of mitochondrial ROS in intestinal tumorigenesis remains controversial. A tumorigenic role for mitochondrial ROS has been, in fact, previously proposed, on the basis of an increase in intestinal tumor initiation and growth in an $APC^{Min/+}$ $Tfam^{+/-}$ mouse, by a mechanism independent of the canonical Wnt pathway [38]. In this animal model, however, ROS would have been involved in the process as major producers of mtDNA damage, rather than as a mediator of the apoptotic cell death. Indeed, oxidative damage to biomolecules is caused by an unbalance between ROS production and scavenging and/or by a defective antioxidant repairing machinery. As well, ROS can act as signaling molecules [39,40] so that a fine tuning of redox biology must be maintained in order to preserve physiological tissue homeostasis.

In conclusion, our study highlights the relationship between APC protein and mitochondrial functions in HT29 colorectal cancer cells. In the presence of wild-type APC, the negative regulation of the Wnt signaling reduces cell survival by promoting apoptosis through the mitochondrial respiratory chain production of ROS. This intriguing scenario adds further insights into the role of mitochondria in intestinal epithelial cell fate and suggests the possibility to target the functional link between Wnt/APC pathway and mitochondrial oxidative metabolism for the therapy and/or prevention of colorectal cancer.

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Transparency document

The Transparencydocument associated with this article can be found in the online version.

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